REVIEW ARTICLE

Decoding *Caulobacter* development

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Abstract

Caulobacter crescentus uses a multi-layered system of oscillating regulators to program different developmental fates into each daughter cell at division. This is achieved by superimposing gene expression, subcellular localization, phosphorylation, and regulated proteolysis to form a complex regulatory network that integrates chromosome replication, segregation, polar differentiation, and cytokinesis. In this review, we outline the current state of research in the field of *Caulobacter* development, emphasizing new findings that elaborate how the developmental program is modulated by factors such as the environment or the metabolic state of the cell.

Introduction

Once, development was thought to be the preserve of eukaryotic multicellular organisms, first distinguishing sister cells from each other and then specifying and differentiating cell lineages that would eventually lead to the entire organism. However, in recent years, it has become clear that similar developmental mechanisms also operate in small bacterial cells, despite their overt simplicity. No longer are they considered as diffusion-limited and disorganized reaction chambers of nucleic acids, proteins, and lipids, but as cells that have impeccably fine-tuned and dynamic regulatory systems that act on a remarkable spatio-temporal scale to implement specialized morphological and functional programs when needed. This plasticity enables bacteria to thrive in all possible niches and respond optimally to fluctuations in their surroundings with developmental programs. Bacterial development may take many multicellular or individual forms, such as sporulation, biofilm formation or asymmetric division, which have been the subject of excellent recent reviews (Lopez *et al.*, 2009, 2010; Shapiro *et al.*, 2009; Errington, 2010; Kaiser *et al.*, 2010). Here, we focus on the newly elucidated mechanisms underlying the asymmetric division of the Gram-negative alphaproteobacterium *Caulobacter crescentus*.

One key aspect of bacterial development is the establishment and maintenance of polarity. Akin to eukaryotic cells, bacteria are able to differentiate the poles from the midcell region, or (in some cases) one pole from another, by localizing polarity determinants which then dictate the development of the appropriate subcellular structures or organelles (Dworkin, 2009). This polarity can be evident at the molecular level even in the absence of visible polar structures, for example in bacteria with seemingly identical poles such as *Escherichia coli* (Maddock & Shapiro, 1993). Because bacteria do not have membrane-bounded compartments in their interior that could be exploited to direct proteins to specific subcellular sites, they have evolved (1) specialized localization mechanisms to direct polarity determinants to the appropriate place, and (2) retention strategies to prevent them from diffusing away (Rudner & Losick, 2010). While several localized polarity determinants have been discovered over the last decade, the mechanisms for their polar positioning are not well understood. One possible mechanism may derive from

the different 'ages' of the poles. At each cell division, the newly forming daughter cells each possess one old pole, from the poles of the mother cell, and one new pole, from the newly incorporated peptidoglycan at the center of the predivisional cell which is constricted at cytokinesis. However, other possibilities also exist, and the nature of the localization signals and the mechanism by which they are interpreted is the subject of intense research.

In the model organism *C. crescentus*, the most evident and best studied developmental strategy relies on an asymmetric cell division (Skerker & Laub, 2004). At every division, the two daughter cells differ from each other in size, morphology, and function (Fig. 1). One, the smaller 'swarmer' cell, possesses a polar flagellum and pili, is motile and capable of chemotaxis but incompetent for chromosome replication. The other is a larger 'stalked' cell which possesses a polar stalk that attaches it to a substrate through a polysaccharide-based holdfast (Bodenmiller *et al.*, 2004; Levi & Jenal, 2006). The stalked cell is capable of chromosome replication, and indeed initiates DNA replication immediately after completion of division, while the swarmer cell must first differentiate into a stalked cell before chromosome replication can be initiated. As outlined in the following paragraphs, this asymmetric division process is highly dependent on the establishment of polarity during every cell cycle. In this review, we will briefly cover the current knowledge about the mechanisms of these regulators and effectors, as these have recently been extensively reviewed (Curtis & Brun, 2010), before turning our attention to the most recent developments in this field and to emerging data on the impact of the environment and the metabolic state on *Caulobacter* development.

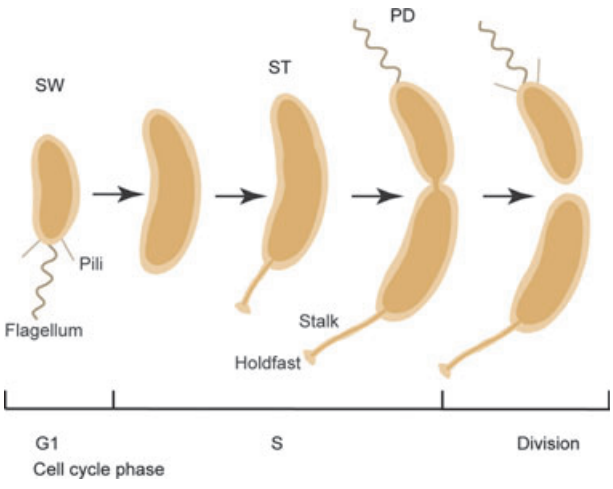

Fig. 1. Asymmetric cell division in *Caulobacter*. SW, swarmer cell; ST, stalked cell; PD, predivisional cell.

Major developmental regulatory pathways of *Caulobacter*

Spatial asymmetry in phosphorylation states

DivK: spatially regulated phosphorylation

The *C. crescentus* genome contains a surprisingly high number of two-component signal transduction genes [105 of 3767 genes at the time of first annotation, (Nierman *et al.*, 2001)], suggesting that these phospho-signaling proteins play a major role in the life cycle of this bacterium. DivK, an essential response regulator, acts as a cell-fate determinant and is regulated by phosphorylation. Phosphorylated DivK (DivK~P, phosphorylated on Asp 53) is found in the stalked cell, while dephosphorylated DivK prevails in the swarmer cell (Jacobs *et al.*, 2001; Matroule *et al.*, 2004). The histidine kinase DivJ that phosphorylates DivK is localized to the stalked pole and is therefore only inherited by the stalked daughter cell. Dephosphorylation of DivK~P is catalyzed by the phosphatase PleC that is sequestered to the flagellar pole and partitions with the swarmer daughter cell. Thus, the daughter cell–specific inheritance of PleC or DivJ dictates which daughter has high levels of DivK~P and which one has low levels (Fig. 2a). Interestingly, DivK not only functions passively as a substrate in this phospho-transfer reaction, but also acts later in the cell cycle as an amplification device for the switch driving the swarmer to stalked transition by directly enhancing the kinase activity of DivJ and converting PleC into a kinase (Paul *et al.*, 2008). PleC kinase activity drives polar remodeling (that is, ejection of the flagellum and development of the stalk and holdfast) through phosphorylation of the diguanylate cyclase PleD (Aldridge *et al.*, 2003; Levi & Jenal, 2006), while increasing DivJ kinase activity rapidly boosts the levels of DivK~P in the cell. These allosteric activities of DivK likely accelerate its own changes in phosphorylation state and program genetic robustness into the system by the formation of a positive feedback loop.

The topology of the DivJ-DivK-PleC phospho-transfer reactions is also influenced by localization factors that direct DivJ and PleC to the appropriate pole. DivJ is recruited to the stalked pole by the muramidase homolog SpmX (Radhakrishnan *et al.*, 2008), while PleC is directed to the swarmer pole by PodJ (Viollier *et al.*, 2002a; Hinz *et al.*, 2003; Lawler *et al.*, 2006). The swarmer-to-stalked transition is accompanied by a sudden rise in DivK~P and a series of ordered polar remodeling events that act on PleC, PodJ, DivJ and SpmX. First, PleC is released from the flagellar pole and degraded along with PodJ (Viollier *et al.*, 2002a, b; Chen *et al.*, 2005). This

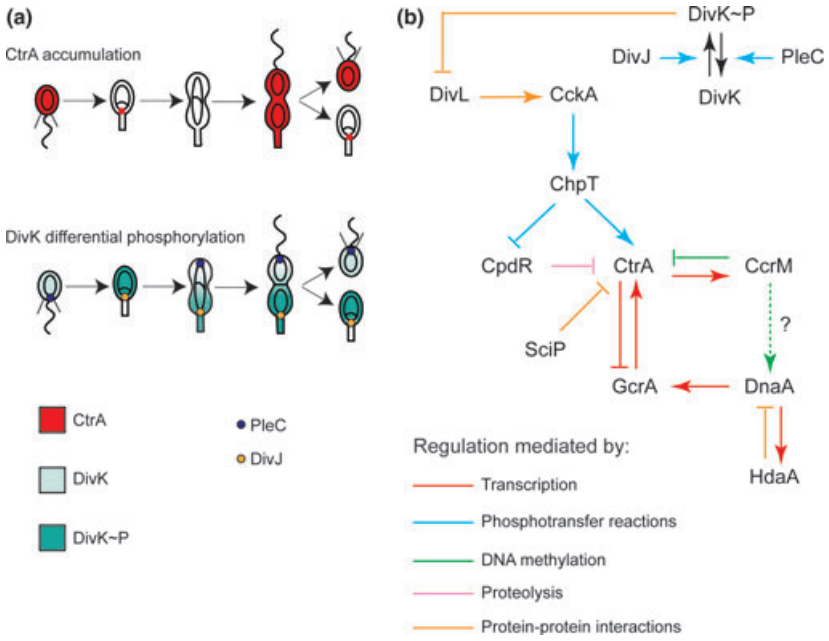

Fig. 2. (a) Cell-type-dependent localization of the master regulator CtrA and cell-type dependent phosphorylation of the cell fate determinant DivK, the major events driving asymmetric development in *Caulobacter*. (b) The genetic circuit model of CtrA, GcrA, DnaA, and CcrM. Dotted lines indicate that the interaction is not fully elucidated; solid lines indicate that the link is confirmed but do not necessarily indicate a direct interaction.

coincides with the acquisition of SpmX and its localization to the same pole. Localized SpmX then recruits and stimulates DivJ, leading to a 'burst' of DivK~P catalyzed by DivJ. Interestingly, the *spmX* gene is upregulated in a PleC-dependent manner prior to the transition, showing that this swarmer pole regulator signals forward to prepare the impending transition to the stalked cell pole (Radhakrishnan *et al.*, 2008). PleC also regulates other developmental events by unknown mechanisms: the ejection of the polar flagellum, the formation of the holdfast and the elaboration of the stalk at the vacated pole, the switch in cell density during the swarmer (more dense) to stalked (less dense) cell transition, and its own release from the pole (Sommer & Newton, 1988, 1989; Wang *et al.*, 1993; Viollier *et al.*, 2002b; Aldridge *et al.*, 2003; Biondi *et al.*, 2006a; Radhakrishnan *et al.*, 2008). The molecular events acting on and affected by DivK illustrate that the regulatory circuit is genetically imprinted to direct the development of the predivisional cell into swarmer and stalked cells, and then differentiation of the swarmer progeny back into stalked cells, in the ensuing division cycle.

CtrA and CpdR: spatially regulated proteolysis

How is the differential phosphorylation of DivK translated into a downstream effect on the developmental cycle? The major transcriptional regulator of *Caulobacter* development, CtrA, is a multifunctional DNA binding protein whose activity and abundance are indirectly influenced by the phosphorylation state of DivK. CtrA is regulated at several levels including transcription, phosphorylation, localization, and proteolysis (Domian *et al.*, 1997). In the swarmer cell, phosphorylated CtrA (CtrA~P) binds to sites near the chromosomal origin of replication (Quon *et al.*, 1998). This interaction, presumably aided by other mechanisms (Cheng & Keiler, 2009; Collier & Shapiro, 2009), prevents premature initiation of DNA replication (Quon *et al.*, 1998). CtrA is degraded at the swarmer-to-stalked transition (Domian *et al.*, 1997), rendering the chromosomal origin of replication competent to fire. CtrA is re-synthesised and (re)-phosphorylated later in the stalked cell phase, and again binds to the replication origin. CtrA~P also binds to promoters of developmental genes to activate or repress their transcription (Laub *et al.*, 2000), oscillating in-phase or out-of-phase, respectively, with CtrA activity over the cell cycle.

The phosphorylation and proteolysis of CtrA is regulated indirectly by DivK~P via the phosphotransfer pathway specified by the hybrid histidine kinase/phosphatase CckA and the histidine phosphotransferase protein ChpT (Biondi *et al.*, 2006a, b). When DivK~P levels are low (in the swarmer cell), CckA is active and sequestered to the pole where it first autophosphorylates and then transfers the phosphate group to ChpT, which is used to phosphorylate CtrA. When DivK~P levels are high (in the

nascent stalked cell), the phosphate flow is reversed: CckA is delocalized and its autokinase activity inhibited. Instead, CckA now acts as a phosphatase, ultimately draining the phosphate from CtrA (Biondi *et al.*, 2006a, b; Chen *et al.*, 2009). Remarkably, the same pathway regulates the phosphorylation state of the single domain response regulator CpdR, which promotes proteolysis as an adaptor protein to the ClpXP protease (Abel *et al.*, 2011) and is required for efficient degradation of CtrA and other proteins *in vivo* (Biondi *et al.*, 2006b; Iniesta *et al.*, 2006). In contrast to CtrA, CpdR is inactive and dispersed when phosphorylated. It is active when de-phosphorylated, localizing to the nascent stalked pole and recruiting ClpXP which degrades CtrA (Jenal & Fuchs, 1998). The ClpXP-dependent degradation of CtrA also seems to involve a second signal input in the form of cyclic-di-guanosine monophosphate (c-di-GMP) that interacts with a receptor protein, PopA, which facilitates CtrA degradation *in vivo* (Duerig *et al.*, 2009). Stalked polar localization of this protein is dependent on cyclic-di-GMP binding, and once localized, it recruits the CtrA-binding protein RcdA (McGrath *et al.*, 2006) and the ClpXP protease for CtrA degradation. Recent data show that the equilibrium between the activity of the DgcB diguanylate cyclase and that of the antagonistic PdeA phosphodiesterase modulates this pathway (Abel *et al.*, 2011).

Genetic circuits: CtrA and transcriptional regulation

The integrity and seamless function of transcriptional circuitry that drives the *Caulobacter* cell cycle and developmental program is dependent on CtrA. CtrA defines a critical transcriptional node within this circuit and as such is essential for viability. As mentioned earlier, it regulates many developmental genes, and the replication origin, but it also tunes its own gradual accumulation over the cell cycle. Transcription of the *ctrA* gene is precisely regulated in space and time by two promoters, P1 and P2, both of which contain CtrA binding sites (Skerker & Laub, 2004). However, the response of each promoter to CtrA binding is different. The P1 promoter is activated, albeit weakly, in late stalked cells, triggering the synthesis of CtrA. This synthesis is self-reinforced with CtrA binding and repressing the P1 promoter, while directly activating the strong P2 promoter at the late predivisional stage to spark a pulse of CtrA production which leads to CtrA accumulation in the swarmer cell (Domian *et al.*, 1999). Although the swarmer cell retains high levels of CtrA~P, this does not lead to continued activation of the P2 promoter after cell division (Quon *et al.*, 1996), suggesting that other factors regulate *ctrA* transcription at other stages of the cell cycle.

One such factor is GcrA (Holtzendorff *et al.*, 2004), a master regulatory protein that is essential for viability and that exhibits a cell cycle oscillation that is out-of-phase with that of CtrA. GcrA is responsible for the transcription of the *ctrA* gene from the P1 promoter in the late stalked cell (Holtzendorff *et al.*, 2004). Another contributor to the timing of *ctrA* transcription is the essential DNA methylase CcrM, which catalyzes methylation of adenine bases in the recognition site GANTC (Zweiger *et al.*, 1994; Berdis *et al.*, 1998). The P1 promoter of *ctrA* is active only in the hemimethylated state (Reisenauer & Shapiro, 2002), which occurs immediately after the DNA replication fork passes through the *ctrA* locus on the chromosome, leaving the DNA hemimethylated. At this stage, the *ctrA* P1 promoter is activated by GcrA, CtrA~P accumulates once again and activates transcription of several genes including *ccrM*. Upon its synthesis, CcrM re-methylates hemimethylated GANTC sites, inactivating the *ctrA* P1 promoter. Thus, CtrA activates transcription of its own negative transcriptional regulator.

The DnaA protein defines another critical node of the cell cycle circuitry (Gorbatyuk & Marczynski, 2001). DnaA is essential for the initiation of DNA replication, while also directly regulating the transcription of many cell cycle genes. As *Caulobacter* replicates its chromosome only once per cell cycle, it is vital that DnaA is tightly controlled in order to prevent re-initiation of a second round of replication before the cycle is completed and the daughter cells divide. DnaA activity is dependent on ATP binding, and hydrolysis of ATP renders DnaA inactive for replication initiation. DnaA is regulated at the post-translational level by the replisome-associated protein HdaA, an inhibitor of DnaA activity (by stimulation of ATPase activity), as a replication initiator protein and perhaps also as a transcription factor (Collier & Shapiro, 2009). DnaA activates HdaA expression (directly or indirectly). Thus, after the peak in its activity, DnaA shuts itself down again by promoting the synthesis of its own inhibitor. DnaA also appears to be regulated at the level of proteolysis (Gorbatyuk & Marczynski, 2001; Grunenfelder *et al.*, 2001).

In addition to tight control of DnaA activity, *dnaA* transcription is cell cycle-regulated, accumulating prior to the onset of DNA replication (Zweiger & Shapiro, 1994; Laub *et al.*, 2000). The *dnaA* gene is located relatively close to the origin of replication and therefore, is among the first genes to be replicated. After replication, the DNA is hemimethylated. It has been proposed that *dnaA* transcription is regulated by methylation of a CcrM-recognition sequence (GANTC) located in the promoter (Collier *et al.*, 2007). Indeed, mutation of the cytosine, although not the critical adenosine, impairs *dnaA* transcription (Cheng & Keiler, 2009). If CcrM-mediated adenosine

methylation directly regulates *dnaA*, then these findings suggest the simple transcriptional regulatory circuit of four sequentially acting master transcriptional regulators with the order: CcrM > DnaA > GcrA > CtrA > CcrM (Fig. 2b).

If regulation of *dnaA* by CcrM is indirect and does not involve adenine methylation of the *dnaA* promoter, then there are still missing links in the circuit. Perhaps *dnaA* is regulated by cytosine methylation, which could explain the observed effect of the key cytosine residue, while preserving the notion of the current model that the activity of the *dnaA* promoter is different in the methylated vs. hemi-methylated state and therefore dependent on DNA replication. Interestingly, at least two putative DNA cytosine methyltransferases, CC1033 and CC3626, are encoded in the *C. crescentus* genome. While their functions remain to be explored, CC1033 does contain one GANTC site in its promoter, suggesting the possibility of a link between adenosine and cytosine methylation. However, the putative dependency of adenosine methylation on abundance or activity of CC1033 and CC3626 could also occur through an indirect route. Thus, chromosome methylation might function as a 'ratchet' to ensure that transcription of cell cycle genes proceeds in an ordered (forward) fashion (Collier *et al.*, 2007). Together, these mechanisms act in a concerted fashion to restrict DnaA activity, to a short window during the swarmer-to-stalked cell transition when DNA replication initiates (Collier *et al.*, 2006). Oscillations in DnaA activity dictate the temporal pattern of DNA replication during the cell division cycle that can act as a 'pacemaker' of DNA replication even in the absence of CtrA, although the periodicity is apparently modulated by HdaA and tmRNA (Keiler & Shapiro, 2003; Collier & Shapiro, 2009; Jonas *et al.*, 2011). The CtrA~P regulatory system is superimposed on the DnaA-controlled replication cycles to impart the spatial asymmetry of DNA replication at cell division (Jonas *et al.*, 2011), ensuring the silencing of the origin of replication in the progeny swarmer cell, while the origin in the progeny stalked cell can fire owing to the absence of CtrA~P.

In addition to its role as a DNA replication initiator, DnaA is a transcriptional regulator of *gcrA* (Collier *et al.*, 2006). This regulation ensures that GcrA accumulates in the replicating stalked cell where the function of the GcrA target genes [encoding DNA replication factors such as RecJ, DnaQ, gyrase A and the ParE subunit of Topo IV, (Holtzendorff *et al.*, 2004)] are needed.

Division plane establishment: MipZ and FtsZ

DnaA also appears to promote early events of cytokinesis by transcriptionally regulating the gene encoding FtsZ (Hottes *et al.*, 2005), a bacterial tubulin homolog that is a conserved mediator of cytokinesis in a wide range of bacteria (Margolin, 2005). FtsZ monomers first polymerize into arcs or ring-like structures at the division plane of the cell. The FtsZ ring then recruits other components of the cell division machinery (the divisome) and is thought to contribute to the mechanical force which constricts the division plane and finally pinches off the two daughter cells from one another (Osawa *et al.*, 2008). However, the regulatory mechanisms by which the division site is chosen and FtsZ positioned there are not so well conserved between bacteria. Two major regulatory mechanisms are the Min system and nucleoid occlusion (not mutually exclusive), where the Min proteins are localized to the cell poles and prevent GTP-dependent FtsZ polymerization there, so that the FtsZ ring only forms at mid-cell, while nucleoid occlusion prevents formation of the FtsZ ring in any region of the cell occupied by chromosomal DNA (Margolin, 2005). However, in *Caulobacter*, the Min system is not conserved, and the FtsZ ring has been observed to form at the division plane before chromosome segregation is complete, implying that nucleoid occlusion is not operating either.

The mechanism employed by *Caulobacter* to regulate FtsZ positioning was identified by Thanbichler & Shapiro (2006) and involves the ParA-like ATPase MipZ. Like ParA, MipZ interacts with the ParB DNA-binding protein, but fulfills a different function. While ParA contributes to chromosome segregation by driving the ParB-bound origin region to the new pole, MipZ forms a bipolar gradient (through binding to ParB) with its maxima at the ParB foci and a minimum at midcell. MipZ stimulates the GTPase activity of FtsZ and thus inhibits polymerization, permitting FtsZ assemblies only near the division plane. This inhibitory mechanism is distinct from that of MinC, the well-studied division inhibitor of *E. coli* which destabilises FtsZ protofilaments without affecting GTPase activity (Hu *et al.*, 1999). At the ultrastructural level *in vitro*, MipZ converts straight protofilament bundles to curved structures, similar to those seen at the ends of eukaryotic microtubules (Tran *et al.*, 1997), which may provide a physical explanation for the inhibition of FtsZ ring formation. Thus, MipZ provides a link between chromosome segregation, through the Par system, and FtsZ-mediated cytokinesis in *Caulobacter*.

Marking the new pole as the future flagellum assembly site: TipN, TipF and PflI

Polar flagellation in *Caulobacter* is intimately linked to cytokinesis as the flagellum is always constructed at the new pole, i.e. the one formed by the most recent division event. The reason for this consistent polarity was

unknown until recently, when the polarity factor TipN was identified (Huitema *et al.*, 2006; Lam *et al.*, 2006). This protein localizes to the pole opposite to the stalk or flagellum in stalked or swarmer cells, respectively, and during the development of the predivisional cell recruits flagellar assembly factors and structural proteins. At cytokinesis (once the flagellum has been assembled), TipN leaves the pole and relocalizes to the divisome through interaction with the Tol-Pal component of the divisome (Huitema *et al.*, 2006; Lam *et al.*, 2006; Yeh *et al.*, 2010; Goley *et al.*, 2011). It remains colocalized with FtsZ as the cell divides, so that it marks the newest pole after division and leads to the formation of the flagellum at the correct pole for the next round of division. TipN therefore acts as a 'birth scar' marker to identify the new pole of newly divided cells. Other factors modulating polar flagellum formation are the TipF assembly regulator (Huitema *et al.*, 2006) and the PflI positioning factor (Obuchowski & Jacobs-Wagner, 2008). These proteins operate downstream of TipN, such that TipF relies on TipN for localization, and PflI in turn depends on TipF. Interestingly, TipF contains an EAL domain (named after the defining glutamate-alanine-leucine signature), which in other proteins can bind and/or hydrolyze the signaling molecule c-di-GMP (Jenal & Malone, 2006). In the case of TipF, the EAL domain acts essentially as a receptor protein as it is incompetent for c-di-GMP hydrolysis. Recent data show that c-di-GMP binding by TipF is a functional requirement for its own polar localization, recruitment of PflI and ultimately flagellum formation (N. J. Davis and P. H. Viollier, unpublished).

New insights into 'hardwired' developmental mechanisms

DivL and CckA: microdomains without membranes

Recent data implicate DNA replication as a trigger for the CckA-ChpT-CtrA/CpdR phosphorelay via the DivL histidine kinase. DivL is essential for viability and was originally identified in a screen for motile suppressors of the *pleC* mutant phenotype which also led to the discovery of the genes encoding DivK and DivJ (Sommer & Newton, 1991). For some time, its role in cell division was mysterious. DivL possesses a tyrosine residue (Y550) instead of a histidine at the catalytic site (Wu *et al.*, 1999) and it appears that the critical functions of DivL in cell cycle control are not dependent on the kinase domain residing in the C-terminal part of the protein (Reisinger *et al.*, 2007). Instead the N-terminal (signal sensing) domain appears to confer the essential activity and it was recently implied that DivL impinges on the CckA-ChpT-CtrA/CpdR phosphorelay by acting on CckA. In an imaging-based screen for mutations which prevent the localization of CckA to the swarmer pole of the predivisional cell, it was found that DivL was required for the localization of CckA and that it stimulated its autophosphorylation. In the absence of DivL, CckA was not localized to the swarmer pole and the phosphorelay was not activated, resulting in the lack of phosphate transfer to CtrA. Again, the DivL kinase activity was dispensable for this function because a Y550F mutation had no effect on CckA localization (Iniesta *et al.*, 2010b). These authors also discovered that DivL and CckA localization to the pole was dependent on initiation of DNA replication (Iniesta *et al.*, 2010b), implying that DivL may be part of a checkpoint which ensures that development of the predivisional cell does not proceed if chromosome replication cannot initiate. The role of DivL and DNA replication is particularly intriguing in light of the possibility that the kinase and phosphatase activities of CckA are confined to opposite poles. This notion has also been incorporated into recent cell cycle models to suggest the existence of phosphogradients of CtrA~P (Chen *et al.*, 2010).

One issue with this model remained unclear until recently, namely why does CckA localize in a dynamic manner when it is neither asymmetrically inherited nor required for regulation of a polarly localized factor? This was resolved by the recent work of Tsokos *et al.* (2011) on the regulatory role of DivL. Here, it was confirmed that DivL is required to localize CckA at the swarmer pole of the predivisional cell, and that DivK is upstream of (and inhibits) DivL. Inhibition by DivK is mediated by direct binding of DivK~P to DivL, so that DivL is inactive and CckA is delocalized from the stalked pole once the DivK kinase DivJ is localized and active there (Fig. 2a). The lowest concentration of DivK~P is at the swarmer pole, because of the presence of the DivK phosphatase PleC, and this study found that PleC activity at the swarmer pole was responsible for DivL and CckA activity there by keeping levels of the inhibitor DivK~P low. Hence, PleC provides a protective 'microdomain' at the swarmer pole in which CckA can activate its downstream phosphorelay, triggering the development of this pole into the swarmer daughter cell. This intricate mechanism provides a way of regulating development by localization to a functionally distinct part of the bacterial cell in the absence of membrane-limited internal compartments.

Yet another layer of regulation of the master regulator CtrA

With the identification of SciP, a small regulatory protein that inhibits CtrA activity and/or transcription of target genes (Gora *et al.*, 2010; Tan *et al.*, 2010), another additional

layer of regulation for CtrA was recently discovered. As described previously, it was not clear why the *ctrA* P2 promoter is inactive in the swarmer cell stage even though CtrA~P is present. SciP is present in the swarmer cell, is quickly degraded at the swarmer-to-stalked transition and accumulates again as the predivisional cell is compartmentalized by the cytokinetic machinery. SciP binds directly to CtrA, disabling CtrA-mediated activation of transcription, while not affecting genes repressed by CtrA. While SciP does not affect DNA binding, phosphorylation or degradation of CtrA, it appears to interfere with the recruitment of RNA polymerase. Consequently, many CtrA-dependent promoters that fire in the predivisional cell (for example those encoding the early flagellar structural proteins, components of the chemosensory apparatus and CtrA itself through the P2 promoter) are inhibited by the accumulation of SciP in the nascent swarmer cell compartment. The fact that *pilA* gene is activated by CtrA in swarmer cells despite the presence of SciP, suggests that it is apparently immune to inhibition by SciP and/or that there are pockets from which SciP is excluded spatially. If this turns out to be true, the underlying mechanism(s) remains to be determined.

The discovery of the SciP regulator provides another compelling example in the paradigm of fine tuning of two component systems by accessory factors. While 'connectors' which link two-component systems into networks have already been proposed (Mitrophanov & Groisman, 2008), it seems that SciP should rather be classed as a modulator because of its selective function on CtrA transcriptional activation. Notwithstanding the appropriate functional definition for SciP, it is clear that we can no longer consider cell cycle phospho-signaling systems of *Alphaproteobacteria* as simple two-state switches (usually phosphorylated = ON, dephosphorylated = OFF) but must take into account further layers of regulation permitting fine tuning akin to a dimmer switch, especially because SciP is conserved in all bacteria that possess a CtrA homolog (Gora *et al.*, 2010). Further analysis of interconnection of transcriptional regulators, including SciP and CtrA, for example by chromatin immunoprecipitation – deep sequencing (ChIP-SEQ) may extend the model of the transcriptional circuit regulating development beyond its current two-dimensional state (Fig. 2).

Interaction of the par chromosome segregation system with the polarity factor TipN

It was recently elucidated how the replicated chromosome is directed poleward to coordinate chromosome partitioning with the *Caulobacter* cell division cycle. The initial studies on TipN showed that in addition to a flagellar placement defect, TipN loss resulted in the misplacement of the division septum to give a larger swarmer cell and smaller stalked cell than is normally observed (Lam *et al.*, 2006), suggesting that TipN might also be involved in cytokinesis regulation. Indeed, it was recently found that TipN interacts genetically and biochemically with the Par chromosome segregation machinery (Ptacin *et al.*, 2010; Schofield *et al.*, 2010). Real-time analysis of FtsZ and MipZ dynamics showed that MipZ (and therefore, the origin of the newly replicated chromosome) travelled more slowly and erratically to the new pole in the TipN mutant, with occasional reverses back toward the old pole. This led to delayed formation and erroneous positioning of the FtsZ ring at a position closer to the stalked pole than is usual, because the MipZ gradient extended further down the cell from the opposite end. Analysis of cells carrying fluorescent fusion derivatives of ParA showed that this effect on MipZ was mediated by the Par system. In wild-type cells, ParA formed a 'cloud' over the nucleoid, consistent with its DNA-binding activity (Gerdes *et al.*, 2010) which retracted promptly to the new pole and remained there for the rest of the cell cycle. In TipN mutant cells, ParA did not retract smoothly to the new pole or accumulate there, and some remained at the old pole (which was never observed in wild type cells). Fluorescence resonance energy transfer (FRET) and pull-down experiments demonstrated that TipN and ParA interacted directly, leading to a model where TipN is proposed to bind and sequester ParA at the new pole as it is released from the DNA-bound 'cloud' thereby preventing it from returning behind the ParB-bound *parS* site and pulling it and the origin to the opposite pole (Schofield *et al.*, 2010) where the ParB-*parS* complex is immobilized and captured by the PopZ polar matrix (Bowman *et al.*, 2008; Ebersbach *et al.*, 2008). Super-resolution fluorescence microscopy recently revealed that the ParA 'cloud' seems to be composed of filamentous linear polymers (Ptacin *et al.*, 2010), which were formed on (non-specific) binding to DNA and depolymerized by ParB. Interestingly, the ParA-mediated movement of the ParB-*parS* kinetochore-centromere (and the origin) is only one part of a recently proposed four-step poleward movement (Shebelut *et al.*, 2010). The four stages are as follows: (1) release of both origins from PopZ (acts as a polar anchor for the chromosome) at the old pole (Bowman *et al.*, 2010), (2) polar retraction of one origin back toward the old pole, (3) early translocation of the other origin (from pole to midcell), and (4) late translocation (from midcell to pole). The Par system was only required for late translocation, which occurred at a significantly faster velocity than early translocation. These observations suggest that while initial origin separation may be by a relatively simple bulk separation mechanism, completion of chromosome segregation in *Caulobacter* is an active and

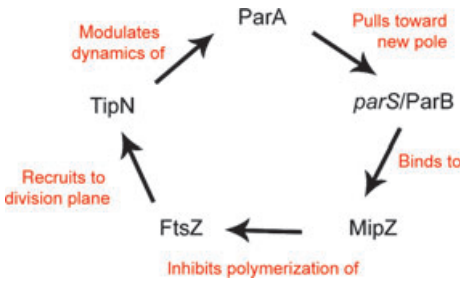

Fig. 3. Feedback loop showing the interaction of the Par system, MipZ, FtsZ and TipN.

multi-phasic process with complex regulation similar to that seen in eukaryotes. They also indicate that a feedback loop consisting of the Par system, MipZ, FtsZ and TipN is an integral part of chromosome segregation and division control (Fig. 3).

New insights into metabolic and environmental influences on development

Extracellular DNA, a kin-specific dispersion signal from *Caulobacter* biofilms

In addition to planktonic growth, *Caulobacter* is capable of forming biofilms. In relatively nutrient-rich environments, the swarmer cells do not disperse but tend to adhere to surfaces near their parents (Siegal-Gaskins & Crosson, 2008). As the swarmer cells go through the swarmer-to-stalked transition to obtain the ultra-adhesive holdfast, the nascent stalked cells bind firmly to the surface within a monolayer of cells that matures into a three-dimensional structure (Entcheva-Dimitrov & Spormann, 2004). The biofilm growth mode, while it enables the bacteria to profit from a readily available source of nutrients, imposes its own challenges on the cells buried in the core (e.g. the decreased availability of oxygen and nutrients). Not surprisingly, biofilm growth is regulated to balance these disadvantages against the advantages. An unprecedented mode of kin-specific biofilm regulation was recently discovered in *Caulobacter* by Berne *et al.* (2010). Unlike other biofilm-forming bacterial species, which incorporate macromolecules such as proteins and DNA into an extracellular matrix (Karatan & Watnick, 2009), and in some cases even require DNA as a structural component of the matrix (Whitchurch *et al.*, 2002), *Caulobacter* employs extracellular DNA (eDNA) as a biofilm dispersal signal. Low-molecular-mass eDNA inhibited the attachment of swarmer cells to the biofilm by binding to the holdfast and preventing its attachment to the biofilm-occupied surface, while it did not displace previously attached stalked cells from the biofilm. eDNA concentration correlated positively with cell death and negatively with biofilm formation, suggesting that the source of the eDNA is death and lysis of cells in the biofilm rather than secretion of DNA fragments from living cells. This hypothesis, however, does not exclude that cell death, induced for example by toxin-antitoxin systems, may be deliberately induced as part of a developmental program. Interestingly, the biofilm inhibitory effect was only observed for *Caulobacter* eDNA, as DNA from other species had no effect on *Caulobacter* biofilms (Berne *et al.*, 2010). Therefore, the biofilm should be unaffected by the presence of unrelated bacteria, while modulating itself specifically according to the density of *Caulobacter* cells. These findings demonstrate that a hitherto unprecedented strategy can favor the motile stage of the cell cycle over the adhesive one.

Metabolic regulation of development

Caulobacter usually differentiates from a swarmer to a stalked cell after a fixed time in laboratory culture, suggesting that this differentiation process is 'hard-wired' and driven by an internal clock. While the constituents of such a potential 'molecular clock' remain to be identified, in the natural oligotrophic environment of *Caulobacter* environmental conditions are also likely to influence the relative length of the developmental stages. In support of this hypothesis, England *et al.* (2010) observed that growth in chemostatically nutrient-limited cultures caused global alterations in gene expression which led to changes in the developmental program. Specifically, nitrogen limitation prolonged the swarmer cell phase, consistent with the aforesaid hypothesis. Interestingly, carbon limitation lengthened the cell doubling time affecting each phase equally, suggesting that there are specific nutritional (metabolic) inputs into the developmental program. In another remarkable example of environmental signaling, Purcell *et al.* (2007) identified blue light as a physical stimulus that impacts the *Caulobacter* developmental program via the LovKR two-component system to fine-tune the adhesive properties of the cell. Carbon starvation was shown in two recent studies to feed into the core cell cycle circuitry driving development and cell division (Boutte & Crosson, 2011; Britos *et al.*, 2011). The master regulator CtrA is downregulated in carbon-starved swarmer cells in what appears to be a SigT-dependent manner, although the mechanism has not been identified (Britos *et al.*, 2011). Meanwhile, the methylase CcrM was found to be under the control of SpoT, the *Caulobacter* ppGpp synthetase induced in response to starvation, and it is hypothesized that downregulation of CcrM under starvation conditions would lead to retention of high

levels of CtrA and low levels of DnaA, instead of the antiphase fluctuations of these regulators which are normally observed (Boutte & Crosson, 2011). Therefore, the core cell cycle regulatory circuit is susceptible to tuning by the availability of sufficient nutrients and, thus, the metabolic status of the cell.

The bifunctional regulatory protein KidO provides another illustrative example of how the cells might tune their developmental program according to their metabolic state (Radhakrishnan *et al.*, 2010). An NAD(H)-binding oxidoreductase homolog, KidO modulates both CtrA~P levels and FtsZ function, contributing to the burst of DivK~P production in the stalked cell by activating DivJ kinase activity, while also regulating the assembly and/or stability of the cytokinetic FtsZ ring. Moreover, KidO abundance is cell-cycle regulated: it is present in the swarmer and late predivisional cell, and is cleared from the cell in the stalked and early predivisional stages when the FtsZ ring forms. Remarkably, the degradation of KidO is catalyzed by the ClpXP protease via the same CckA/ChpT/CpdR pathway that regulates the stability of CtrA.

In addition to regulation at the level of protein stability, there is evidence for another level of post-translational regulation for KidO. While KidO can bind NADH, it lacks the catalytic residue required for NADH-dependent oxidation–reduction reactions. Mutations that disrupt the NADH-binding pocket of KidO prevent the FtsZ-inhibitory activity, while the DivJ positive regulation was unaffected. As the NADH-binding capacity of KidO is necessary for one of its functions (Radhakrishnan *et al.*, 2010), the possibility exists that *Caulobacter* uses KidO to gauge cellular NADH levels to regulate cytokinesis depending on the energy level of the cell. Reminiscent of such a potential signaling role of NAD(H) in *Caulobacter*, eukaryotic cells are also thought to use metabolites such as NAD(P)H to signal cyclic processes such as the yeast cell cycle or the mammalian circadian clock (Tu & McKnight, 2006; Tu *et al.*, 2007; Asher *et al.*, 2008) even in the absence of transcription and translation (O'Neill & Reddy, 2011; O'Neill *et al.*, 2011).

Regulated cell death through toxin–antitoxin systems

Toxin–antitoxin (TA) systems, first discovered as plasmid-encoded genes, function as retention systems to kill off plasmid-free cells and ensure stable inheritance of the plasmid (Gerdes *et al.*, 1986). However, with the advent of whole-genome sequencing, many chromosomally encoded TA systems have been discovered, frequently as multiple paralogous copies (Pandey & Gerdes, 2005). Three types of TA system have been characterized to date, which differ in the form and function of the antitoxin. While the toxins of these systems are always proteins, type I antitoxins are cis-acting antisense mRNAs, while type II antitoxins are proteins. Type III TA systems have only recently been discovered and their antitoxins function as protein-binding RNAs rather than antisense RNAs (Fineran *et al.*, 2009). Type II TA systems (the others will not be further discussed here) are two-gene operons, usually translationally coupled, with the antitoxin gene preceding the toxin gene and often with the antitoxin acting as a repressor of its own transcription (Fig. 4). In unstressed cells, the antitoxin forms a complex with the toxin, preventing it from acting on its targets within the cell. Under stressful conditions, the antitoxin is degraded by proteases, freeing the toxin to act and relieving the transcriptional repression of the operon. In the case of TA systems for plasmid maintenance, the two daughter cells inherit the TA complex, but because the antitoxin protein is usually less stable than the toxin, cells can only replenish the antitoxin if they retain the plasmid. Plasmid-free cells are killed upon release of the toxin, leading to stable maintenance of the plasmid in the population (Hayes, 2003). Killing is mediated through mRNA cleavage at the ribosome by RelE-family toxins (Neubauer *et al.*, 2009), or DNA gyrase inhibition by ParE-family toxins (Jiang *et al.*, 2002).

The multiplicity of these inducible self-killing genes on bacterial chromosomes suggests that they may be used for executing controlled cell death as part of a developmental program (Engelberg-Kulka *et al.*, 2006). *Caulobacter* possesses 11 chromosomal type II TA systems, of which the

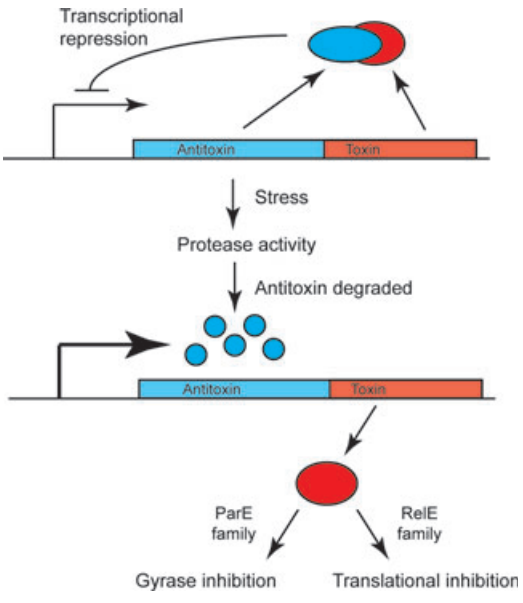

Fig. 4. The classical paradigm of type II TA systems of the Par and Rel family (other systems are not reviewed here).

functions are by and large not well understood. Recent work by Fiebig *et al.* (2010) on the RelBE and ParDE homologs of *Caulobacter* provides genetic evidence that these systems form insulated units so that each toxin interacts only with its co-encoded antitoxin and there is no cross-talk between systems, even when the antitoxin genes are artificially overexpressed, consistent with the idea that they are active under specialized conditions. Furthermore, transcription of the systems was differentially regulated in response to various environmental stressors such as oxidative stress and heat shock. Some of the operons were also transcriptionally upregulated in mid log growth phase relative to early log growth, in the absence of any stress, implying a possible role for TA systems in the natural progression of the *Caulobacter* life cycle, although this is not yet confirmed. It might be interesting to investigate whether there is cross-talk between TA systems and other developmental regulatory factors at the level of transcription (or elsewhere). Indeed, it has been observed that the promoters of some of the *Caulobacter* TA systems are bound by the SOS (DNA damage) response regulator LexA (da Rocha *et al.*, 2008) (Radhakrishnan and Viollier, unpublished), suggesting that while they may be insulated from cross-talk with each other they can be integrated into genetic or developmental control circuits.

Conclusions

Studies of the bacterial cell cycle in *Caulobacter* have unmasked many regulatory mechanisms not observed in model systems with apparently symmetrical division. With the recent developments reviewed herein, additional levels of complexity have surfaced to an already intricate cell differentiation process in a so-called 'simple' bacterial cell. This progress is attributable in part to the rapid improvement of analytical methods that have fueled these discoveries, above all the methods for single-cell level fluorescence imaging. FRET, fluorescence loss in photobleaching and fluorescence recovery after photobleaching strategies especially have enabled *in vivo* confirmation of molecular interactions that could previously only be observed *in vitro*. Moreover, as the limits of resolution of fluorescence microscopy decrease by improved optical and computational methods, it is becoming possible to observe new processes at the submicron level. For example, high-resolution RNA localization experiments recently suggested that transcripts are immobile, localizing to the corresponding position in the cell where the gene is located, and that they capture the much larger ribosomal particles that diffuse by (Montero Llopis *et al.*, 2010), with tRNAs presumably posing an exception to this restricted diffusion of transcription. Moreover, a single-cell-based FRET sensor (Christen *et al.*, 2010) confirmed the notion that the signaling molecule c-di-GMP is differentially partitioned at cell division with higher concentrations found in the *Caulobacter* swarmer cell than in the stalked cell (Paul *et al.*, 2008). Future research using high-resolution microscopic methods will uncover new regulatory pathways that are confined in subcellular space and/or as a function of cell cycle as is the case in eukaryotes (Dehmelt & Bastiaens, 2010).

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